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Opponent Activities of Shh and BMP Signaling during Floor Plate Induction In Vivo

Iain Patten and Marysia Placzek[1]
Centre for Developmental Genetics
School of Medicine and Biomedical Science
University of Sheffield
Sheffield, S10 2TN
United Kingdom

Summary

We performed in vivo experiments in chick embryos that examined whether application of an exogenous source of Shh protein mimics the ability of the notochord to induce ectopic floor plate cells in the neural tube. Shh cannot act alone to induce a floor plate. However, coapplication of Shh and chordin, a BMP antagonist normally coexpressed with Shh in the notochord, results in a marked switch from dorsal to ventral cell fate, including a dramatic and widespread induction of floor plate cells. These data provide in vivo evidence that notochord-derived BMP antagonists may normally generate a permissive environment for the Shh-mediated induction of floor plate. Further experiments performed to address the source of BMPs that are inhibited by the action of chordin suggest that they derive specifically from the surface ectoderm and dorsal-most neuroepithelium. These data indicate that, at neural groove stages, dorsally derived BMPs affect ventral-most regions of the neural plate, suggesting a novel long-range action of BMPs. Together, these studies suggest that the balance of dorsally derived signals and notochord-derived signals determines the extent of floor plate cell induction.

Results and Discussion

In the embryonic chick, the induction of floor plate cells depends on signals from the notochord [1]. Expression of the secreted signaling molecule Sonic hedgehog (Shh) in the notochord is required for its ability to induce floor plate cells. Function-blocking antibodies directed against Shh prevent the notochord from inducing the floor plate [2]. Mice genetically null for Shh, or Shh signaling components, fail to form a floor plate [3–5].

Ectopic expression of Shh and Shh-signaling components in vivo can lead to the induction of ectopic floor plate cells [6–9]. Similarly, exposure of neural cells to Shh protein in vitro results in floor plate induction, suggesting that high levels of Shh can act alone to induce the floor plate [10, 11]. In the embryo, the ventral midline of the neural tube may be exposed to high levels of Shh, due to the close apposition of the notochord [12]. However, none of these observations can establish that the levels of Shh in the notochord are sufficient to independently induce the floor plate or that the intact neural environment is responsive to an exogenous source of Shh. For instance, no study has yet addressed whether ectopic application of Shh, as a point source next to the posterior neural tube, can induce floor plate cells.

To determine this, we compared the ability of the notochord and Shh to induce floor plate cells in vivo. In control experiments, notochord grafts induced ectopic floor plate cells expressing Shh and HNF3β (n = 8, Figures 1A and 1B). Expression of Pax 6 and Pax 7 was downregulated, and ectopic Islet1 +ive motor neurons were detected (Figures 1C–1E). In contrast, implants of Shh-N-soaked beads (n = 20, data not shown) or cells transfected with full-length Shh (n = 25) were unable to affect neural tube cell pattern (Figures 1F–1J). When identically prepared beads and cells were cultured with intermediate neural explants in vitro, both Shh-N and Shh induced floor plate cells (n = 6 each, see the Supplementary Material available with this article online). These experiments indicate that, although exceeding the threshold requirement for floor plate induction in vitro, Shh provided as a point source external to the neuroepithelium is unable to induce floor plate differentiation in vivo. This inability of Shh to mimic the notochord suggests that notochord-mediated floor plate induction depends on other factors in addition to Shh.

A number of lines of evidence suggest that floor plate induction can be influenced by BMP antagonists [13–16]. However, the two BMP antagonists particularly implicated in floor plate differentiation, noggin and follistatin, are not expressed in the st-10 caudal notochord ([15] and unpublished data). Furthermore, no study has ascertained whether ectopic application of a BMP antagonist adjacent to the neural tube can affect floor plate development in vivo.

We therefore assayed the ability of chordin, a BMP antagonist coexpressed with Shh in the st-10 caudal notochord (Figures 2N and 2O), to induce floor plate differentiation. Although chordin-soaked beads did not induce floor plate cells in vitro (n = 7, Supplementary Material), they caused a mild dorsal-to-ventral shift in cell pattern in the ipsilateral neural tube when implanted in vivo (n = 6, Figures 2A–2E). Expression of Shh and HNF3β was expanded, the ventral boundary of Pax 6 was shifted dorsally, and motor neurons differentiated in ectopic dorsal positions. Noggin implants were unable to affect neural tube cell pattern (n = 10, data not shown). These observations show that chordin cannot induce floor plate cells but can lead to an increase in the size of the endogenous floor plate. One explanation of these results is that increasing the concentration of chordin can sensitize neuroepithelial cells to notochord-derived Shh, extending the local differentiation of floor plate cells.

To test the idea that chordin may facilitate the ability of Shh to induce the floor plate, beads soaked in Shh and chordin (Shh/Chd) were implanted (n = 7, Figure 2K). The combination of Shh/Chd exerted a remarkable effect on neural tube cell pattern. Expression of Pax 6 and Pax 7 was restricted to the extreme dorsal neural tube, or was abolished (Figures 2I and 2J). Floor plate

[1] Correspondence: m.placzek@sheffield.ac.uk

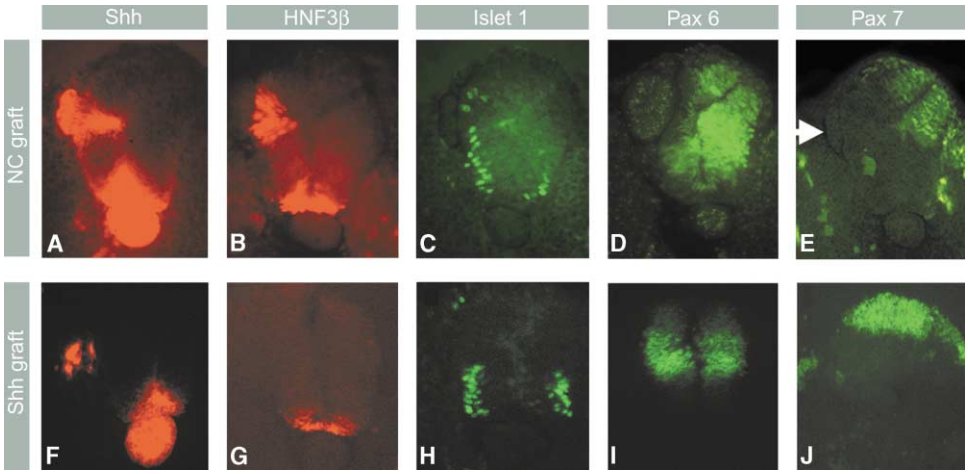

Figure 1. Notochord-Mediated Induction of Floor Plate Is Not Mimicked by Shh

(A–E) A notochord graft (arrow in [E]) induces (A and B) ectopic floor plate cells and (C) motor neurons and represses (D and E) dorsal markers. In a representative section, Pax 7-expressing cells extend 5 cell diameters further ventrally on unoperated compared with operated sides (36% reduction). The high-expression domain of Pax 6 is absent on operated sides (an 8-cell diameter loss; 35% reduction).

(F–J) A graft of Shh-expressing cells (visible in [F]). All domains of marker gene expression show similar dorsoventral limits on operated and unoperated sides. The ventral expression boundaries of Pax 6 and Pax 7 remain intact (Figures 1I and 1J); neither ectopic floor plate cells nor ectopic motor neurons are detected (Figures 1F–1H).

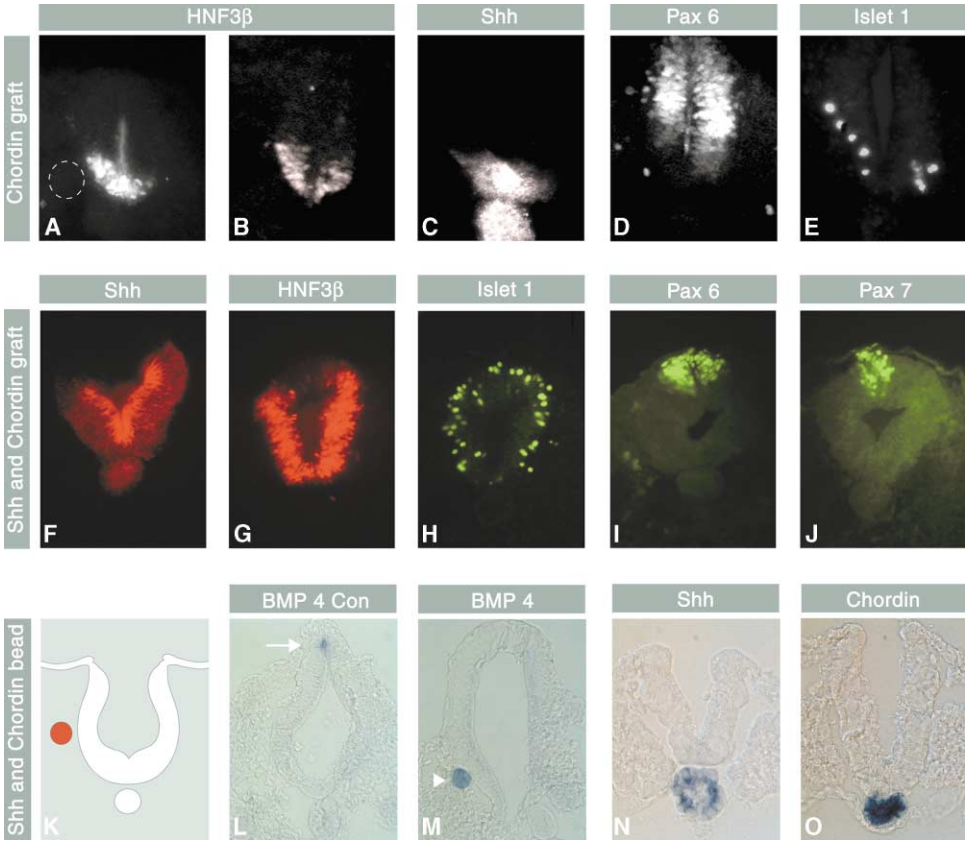

Figure 2. Induction of Floor Plate Cells in Response to Shh and Chordin In Vivo

(A–E) A chordin graft (outline marked in [A]) results in a unilateral expansion of the floor plate ([A–C]; 5-cell diameter expansion of HNF3β cells [42%]) and a coincident dorsal shift in ventral markers ([D and E]; 5-cell diameter dorsal shift in Pax 6 expression).

(F–K) (K) Shh/Chd grafts result in the induction of (F and G) floor plate cells and (H) motor neurons and the restriction of (I and J) Pax 6 and Pax 7 to dorsal-most regions of the neural tube. Floor plate cells occupy ~75% of the neural tube.

(L and M) The normal expression of BMP4 (arrow in [L]) is lost in the vicinity of Shh/Chd-soaked beads (arrowhead in [M]).

(N and O) Coexpression of (N) Shh and (O) chordin in the HH st-10 caudal notochord.

cells expressing Shh and HNF3β were detected bilaterally throughout the ventral and intermediate neural tube, even extending into dorsal regions (Figures 2F and 2G). Bilateral columns of Islet1 +ive motor neurons were likewise detected ectopically, extending as a single line of cells into the dorsal-most neural tube, a region normally occupied by the roof plate (Figure 2H). The bilateral ventralization of the neural tube was accompanied by the suppression of dorsal roof plate cells, as evidenced by the loss of expression of BMP4 in operated regions (Figures 2L and 2M). The contrast between the inability of Shh to induce floor plate cells and the dramatic effect mediated by Shh/Chd provides evidence that the two signals operate together to affect floor plate development. The dorsal-ventral shift in character shows that the effects of Shh/Chd are not to increase cell proliferation or reduce cell death in the floor plate, but to mediate the induction of floor plate cells at the expense of more dorsal cell types.

In contrast to the marked effects of Shh/Chd, Shh/noggin-soaked beads did not repattern the neural tube (n = 10, data not shown). Thus, in chick embryos, we observe the reciprocal effects to those observed in mouse embryos, where chordin null mice appear to show no defects in floor plate differentiation [17], yet noggin null mice noggin lack floor plate cells in the caudal neuraxis [13]. It is possible therefore that, in different species, different combinations of BMPs and BMP inhibitors affect floor plate differentiation.

Our experiments suggest that BMP signaling may normally antagonize floor plate induction in vivo. Members of the BMP family have previously been shown to antagonize both the ventralizing activity of Shh and the differentiation of floor plate cells [15, 18–20]. However, the question of whether such antagonism plays a role in shaping the normal floor plate has remained unresolved (also see discussions in [21, 22]). The unilateral expansion of floor plate cells that we detect in response to a chordin implant suggests that BMP signaling does operate in ventral regions of the forming neural tube and that the balance of BMPs and chordin dictates the size of the endogenous floor plate.

To better understand the tissue interactions that influence floor plate development, we sought to identify the source of BMPs that are inhibited by notochord-derived chordin. *BMP*s have been detected in the dorsal neuroepithelium, the adjacent nonneural ectoderm, and the tail bud of st-10 embryos [19]. Low-level expression of *BMP*s may additionally be present throughout the neural plate [15]. Any of these sources could contribute to ambient BMP levels in the ventral neural tube and could hence affect the lateral extent of the floor plate.

We first examined whether chordin acts similarly to follistatin, increasing net Shh activity by counteracting residual BMPs in the intermediate neural plate [15]. Intermediate neural explants were exposed to a concentration range of Shh with or without the addition of chordin, and the number of floor plate cells and motor neurons was quantified (Figure 3A). Chordin appeared unable to modulate the effect of Shh in inducing ventral cell types. Neither the cell type induced in response to Shh nor the frequency of induction were affected by chordin (Figure 3A). Thus, chordin does not appear to sensitize neural

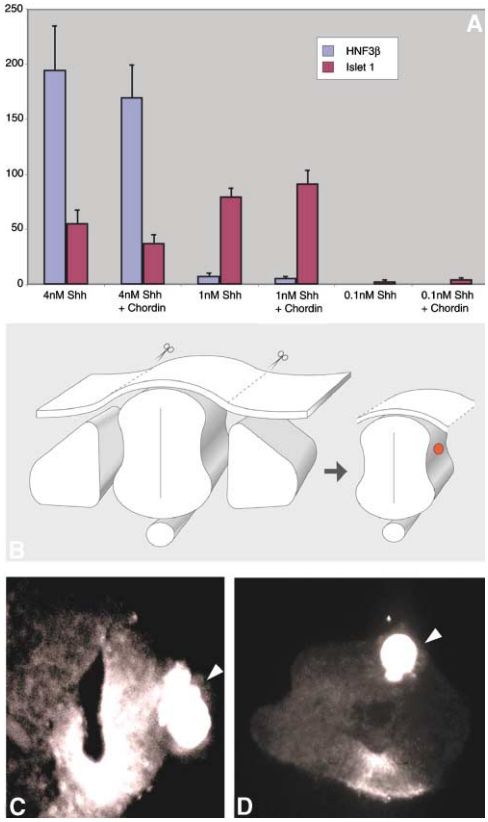

Figure 3. Chordin Does Not Act Directly on Intermediate Regions of the Neural Groove or Indirectly via Somitic Mesoderm/Tail Bud

(A) The addition of 4 nM Shh to explants of intermediate neural plate results in the induction of HNF3β+ cells (195 ± 40) and Islet1 +ive cells (56 ± 12). The addition of chordin during culture does not result in a significant change in the number or ratio of HNF3β- and Islet1-expressing cells (170 ± 30 and 38 ± 8, respectively). Similarly, there is no significant difference between the effect of 1 nM Shh and 1 nM Shh plus chordin in the induction of HNF3β (8 ± 3 and 6 ± 2, respectively)- or Islet1 (80 ± 8 and 92 ± 12, respectively)-expressing cells. The addition of 0.1 nM Shh generates only small numbers of Islet1 +ive cells with (5 ± 2) or without (3 ± 2) chordin. (B–D) Explants comprising neural tissue, axial mesoderm, and surface ectoderm, but not presomitic mesoderm or tail bud, cultured with Shh/Chd beads or Shh beads. (B) Shh/Chd induces ectopic Shh +ive floor plate cells, whereas Shh alone does not (D).

cells to Shh in the same manner as follistatin, by counteracting BMPs resident in the intermediate neural plate.

We next addressed whether BMPs that derive from somites or tail bud antagonize floor plate induction. Explants composed of neural tissue, surface ectoderm, and notochord, in the absence of somites and tail bud, were isolated, and Shh beads or Shh/Chd beads were cultured next to the neural component (Figure 3B). Shh/Chd beads, but not beads soaked in Shh alone, induced ectopic floor plate cells (n = 7 each, Figures 3C and 3D). These results show that the failure of Shh to induce floor plate cells is not due to the action of somite-derived or tail bud-derived inhibitory factors. Instead, they suggest that chordin operates to counteract long-range BMP signaling from either surface ectoderm, dorsal neural tissue, or both.

To test this directly, surface ectoderm and dorsal-

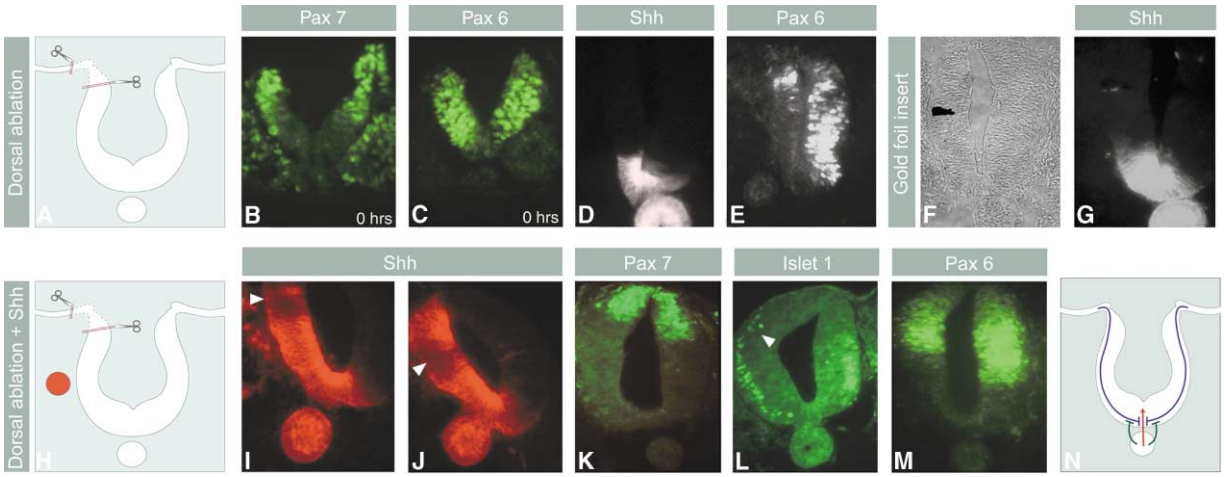

Figure 4. Dorsal Signals Exert Long-Range Effects on Floor Plate Expansion and Inhibit Shh-Mediated Floor Plate Induction

(A–E) Transverse sections showing ablation of ~5–7 cells of dorsal-most neural tissue and adjacent surface ectoderm, as assessed by Pax 7, Pax 6, and Msx 1/2 expression ([B and C] and data not shown). Ablations result in a unilateral expansion of (D) Shh +ive floor plate cells and ([E] and data not shown) dorsal restriction of ventral markers.

(F and G) Insertion of gold foil between basal and alar plates (black sliver in [F]) to prevent diffusion or relay of dorsal signals results in the ipsilateral expansion of Shh +ive floor plate cells (G).

(H–M) The addition of Shh beads to (H) dorsal-ablated embryos results in the ipislateral induction of the (I and J) floor plate. Gaps in the expression of floor plate markers (arrowheads in [I] and [J]) indicate that expansion of floor plate territory is unlikely to be due to a selective proliferation of floor plate cells. Coincident with the induction of the floor plate, the ventral limits of Pax 6 and Pax 7 shift dorsally (K and M), and ectopic Islet1 +ive cells are generated (L).

(N) A model for the signaling pathways that determines the extent of floor plate induction.

Our experiments suggest that, at neural groove stages of development, BMPs from surface ectoderm and the prospective roof plate are relayed to ventral-most regions of the neuroepithelium, where they would prevent the induction of floor plate cells (blue lines). BMP antagonists such as chordin, secreted from the notochord (green lines), inhibit the action of BMPs, creating a competent zone for the induction of floor plate cells by notochord-derived Shh (red arrow).

most neural tube were unilaterally ablated in vivo (Figure 4A). Analysis of embryos fixed immediately postoperatively revealed that a thin wedge of cells could be reproducibly ablated (n = 5, Figures 4B and 4C and legend). In identically treated embryos developed to Hamburger-Hamilton (HH) st 19–21 (n = 6), ablations resulted in the ipsilateral spread of the floor plate (Figure 4D) and a concomitant reduction in Pax 6 expression (Figure 4E). Similarly, insertion of a piece of gold foil into one side of the neural tube to prevent the spread of dorsal signals resulted in the ipsilateral spread of the floor plate (n = 5, Figures 4F and 4G). Thus, signals from the surface ectoderm/dorsal neural tube normally antagonize the lateral extent of the floor plate. Although we cannot rule out that these regions contribute additional inhibitory factors, taken together with our previous results, the most likely explanation is that long-range BMP signaling determines the extent of floor plate differentiation.

Finally, to establish that such long-range BMP signaling normally antagonizes Shh-mediated floor plate induction, Shh-N-soaked beads or Shh-transfected cells were implanted adjacent to intermediate regions of the ipsilateral neuroepithelium in similar tissue-ablated embryos (n = 5 each, Figure 4H). Operated embryos showed a pronounced ventralization of the ipsilateral neural tube. Floor plate cells (Figures 4I–4J) and motor neurons (Figure 4L) differentiated in ectopic positions, and Pax 6 and Pax 7 were repressed (Figures 4K and 4M). Together, these analyses show that Shh-mediated floor plate induction is antagonized by long-range effects from the dorsal edges of the closing neural groove and surface ectoderm. Such a mechanism may antagonize the homeogenetic induction of floor plate that has been proposed to operate in vivo [23].

Together, our experiments suggest a novel, long-range action of BMPs. This finding contrasts with previous studies that show little or no evidence for long-range signaling from the roof plate subsequent to its physical or genetic removal [24, 25]. The most likely explanation for the different observations is due to the timing of the ablations. In our experiments, dorsal tissues were ablated in the region of the caudal neural groove, rather than the neural tube. Our experiments suggest that, at early stages, BMPs that derive from the surface ectoderm both act locally to initiate dorsal neural patterning [19] and exert long-range effects to repress ventral pattern. The mechanism of such long-range BMP signaling remains unclear. BMP proteins may diffuse directly through the neural tube, with chordin regulating BMP activity through a direct interaction of the two proteins [15, 26, 27]. Since the dorsoventral extent of the neuroepithelium at neural groove stages is approximately 30 cell diameters, a distance greater than BMPs appear to travel by simple diffusion [28], such a mechanism may depend on the specialized transport of the signaling protein [29, 30]. Alternatively, the observation that BMPs can activate their own expression [15] raises the possibility that the long-range effects are mediated by a cascade of dorsal-ventral *BMP* expression, initiated and maintained from the surface ectoderm and dorsal

neural tube. In this case, chordin may downregulate *BMP* expression. Previous experiments support the idea that BMP antagonists control *BMP* expression; in noggin null mice, *BMP4* is ectopically expressed in the ventral midline [13], while, in chick embryos, ectopic application of noggin downregulates *BMP4* in the roof plate [31]. Since Shh can also downregulate *BMP*s [19], both Shh and BMP antagonists could contribute to the regulation of such a cascade. To date, however, we do not know of a candidate *BMP* expressed in the neural tube that could be controlled in the proposed manner.

In conclusion, many experiments have suggested an antagonism between dorsally derived BMPs and ventrally derived Shh that is important in patterning intermediate regions of the neural tube. However, the range of action of both Shh and BMP signaling has not been thought to extend throughout the neural tube. Recent functional analysis of the Shh receptor, Patched, suggests that Shh signaling may operate directly over long distances throughout much of the dorsoventral extent of the neural tube [32]. The experiments described here suggest similarly that BMP signaling may exert an effect throughout the dorsoventral extent of the forming neural tube, limiting floor plate expansion (Figure 4N). Together, these observations suggest that the antagonism between dorsal and ventral midline signaling pathways may be important, at neural groove stages of development, for the general patterning of the neural tube, so that the interplay of long-range BMP signaling, BMP antagonists, and Shh may determine the extent of induction not only of the floor plate, but of other ventral cells.

Supplementary Material

Supplementary material including the Experimental Procedures and a figure showing the effects of Shh and chordin in vitro is available at http://images.cellpress.com/supmat/supmatin.htm.

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